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A *cis*-acting element and a *trans*-acting factor involved in the wound-induced expression of a horseradish peroxidase gene

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Summary

The mechanisms that control the wound-induced expression of the *prxC2* gene for horseradish peroxidase (HRP) have been investigated. Analysis of the regulatory properties of 5'-deleted promoters showed that a positive element involved in the response to wounding was located between −307 and −99 bp from the site of initiation of translation. In *in vitro* binding assays of tobacco nuclear proteins and DNA fragments of *prxC2* promoter, the binding site was the Box 1 from −296 to −283 containing the CACGTG motif. To identify the functional role of Box 1, the *prxC2* promoter that has been digested from the 5' end to −289 with a disrupted Box 1 was fused to a reporter gene for β-glucuronidase (GUS). No induction of GUS activity was observed in transgenic tobacco plants with the *prxC2* (−289)/GUS construct. These data indicated that the expression of *prxC2* in response to wounding required the Box 1 sequence from −296 to −283. Furthermore, a tobacco cDNA expression library was screened and a cDNA clone for a protein, designated TFHP-1, that bound specifically to the Box 1 sequence was identified. The putative TFHP-1 protein contains a basic region and leucine zipper (bZip) motif and a helix–loop–helix (HLH) motif. The mRNA for *TFHP-1* was abundant in roots and stems, and it was not induced by wounding in leaves. In tobacco protoplasts, antisense *TFHP-1* suppressed the expression of *prxC2* (−529)/GUS.

Introduction

Plant peroxidases (EC 1.11.1.7; donor:hydrogen-peroxidase oxidoreductase; POD) are glycoproteins. Higher plants contain a number of peroxidase isozymes whose patterns of expression are ogan-specific, developmentally and environmentally regulated (Lagrimini and Rothstein, 1987). Several physiological roles for peroxidases in plants have been suggested, such as the removal of hydrogen peroxide, the oxidation of toxic reductants, the biosynthesis and degradation of lignin (Grisebach, 1981), the catabolism of auxin (Hinnman and Lang, 1965) and defensive responses to wounding (Espelie *et al.*, 1986).

Wounding leads to dramatic physiological changes in plants. Several wound-induced genes have been studied by functional anlaysis of their 5'-promoter regions in transgenic plants, for example, the gene for phenylalanine ammonia-lyase (Ohl *et al.*, 1990), chalcone synthase (Doerner *et al.*, 1990), proteinase inhibitors (Keil *et al.*, 1990), pathogenesis-related proteins (Ohshima *et al.*, 1990) and β-fructosidase (Stum and Chrispeels, 1990). Peroxidase activity increased in response to wounding in tobacco (Lagrimini *et al.*, 1987). In all the cases cited, induction of the protein is accompanied by increased levels of the corresponding mRNA, an indication that regulation of expression occurs at the transcriptional level. Transcriptional regulation involves interactions between nuclear transcription factors, some of which bind to specific *cis*-elements. Many such sequences and their respective binding proteins have been identified (e.g. Lam *et al.*, 1989; Schulze-Lefert *et al.*, 1989) and several genes that encode binding proteins have been isolated (e.g. Katagiri *et al.*, 1989; Oeda *et al.*, 1991).

We have cloned four genomic DNAs that encode HRP, namely, *prxC1a*, *prxC1b*, *prxC2*, and *prxC3*, as described previously (Fujiyama *et al.*, 1990). cDNAs corresponding to *prxC1a*, *prxC1b* and *prxC1c* have also been cloned (Fujiyama *et al.*, 1988). The *prxC2* gene encoding a basic isozyme of HRP is induced at the transcriptional level in horseradish by wounding and GUS activity was induced by wounding in transgenic tobacco that contained a chimeric gene composed of the 5'-promoter region of *prxC2* and the structural gene for GUS (Kawaoka *et al.*, 1994). Here, we describe the functional analysis of the *prxC2* promoter that is involved in determining the extent of wound-induced expression of the gene by 5'-deletion and a DNA-protein binding assay *in vitro*. We also report the isolation of a cDNA clone for a protein, designated TFHP-1, that bound specifically to a specific *cis*-element from a cDNA expression library derived from wounded tobacco leaves.

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Results

Functional analysis of the 5'-non-coding region of prxC2 *in transgenic tobacco*

The sequence of the 5'-non-coding region of *prxC2* has been reported previously (Kawaoka *et al.*, 1992). The functional properties of the promoter were examined by analysis of GUS activity in transgenic tobacco plants that contained the sequence of *prxC2* from –1035 to –99 (numbering relative to the site of initiation of translation) and had been fused to the structural gene for GUS, as a reporter. Figure 1 shows the results for three independent clones of each construct (–1035, –527, –307 and –99 bp). High levels of GUS expression were given by –1035 2 days after wounding in leaves and the 5' deletions to –527 and –307 also allowed induction of GUS activity. However, no significant GUS activity was observed with only –99 bp of *prxC2*. Therefore, the *cis*-element of *prxC2* that is responsive to wounding is located between –307 and –99.

Cis-element of the 5'-non-coding region of prxC2 *for wound-induced expression*

To investigate the nuclear factors that control the expression of the *prxC2* gene after wounding, we searched for nuclear factors that interacted with the 5'-non-coding region of the gene in a gel-retardation assay, using nuclear extracts from unwounded and wounded tobacco leaves. The DNA fragment of the 5'-non-coding region from –307 to –1 was used. As shown in Figure 2, the retarded bands in the presence of nuclear extracts from wounded leaves were more intense than those from unwounded leaves (lanes 2 and 3). In the presence of excess amounts of the homologous unlabeled DNA fragment as competitor, no retarded complexes were observed (lanes 4 and 5). However, addition of pUC19 as competitor gave the same patterns as poly(dI–dC) (lanes 6 and 7). These results suggest the presence of nuclear factors that specifically bind to the 5'-non-coding region from –307 to –1 of *prxC2* upon wounding of leaves.

DNase I footprinting analysis was carried out to characterize the sequences of the nuclear factor-binding sites. A binding reaction with a nuclear extract from wounded leaves and the DNA fragment from –529 to –1 was incubated for 30 min on ice. After treatment with DNase I, the products of enzymatic digestion were subjected to electrophoresis on a denaturing polyacrylamide gel. Figure 3 shows that three different sequences were protected to varying extents from digestion by DNase I. Because crude nuclear extracts were used, digestion patterns were not very clear well. The protected sequences were located at –296 to –283 (Box 1), –257 to –238 (Box 2) and –223 to –212 (Box 3) (Figure 3c). Box 1 had the sequence CACGTG. Box 2 contained three repeats of the GAAA motif and Box 3 was rich in AT sequences.

Since DNase I footprinting revealed that three boxes were protected from digestion by DNase I, we synthesized

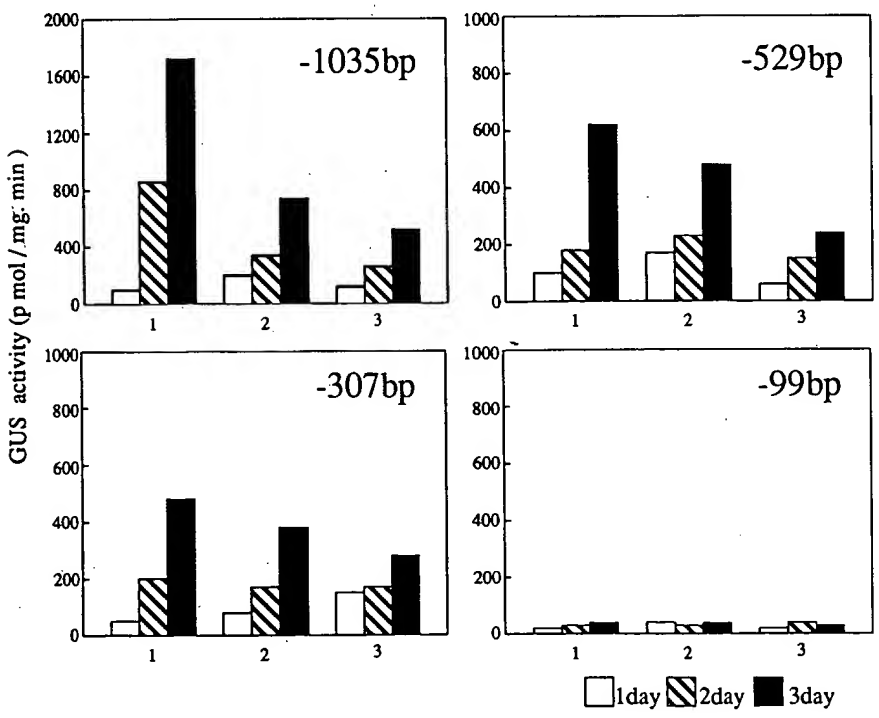

Figure 1. Effect of 5' deletions on the wound-induced expression of the *prxC2*–GUS fusion genes in transgenic tobacco leaves.
Numbers of the abscissa indicate individual transformants.

oligonucleotides that correspond to each of these sequences. Competition experiments in combination with the above gel-retardation assay were carried out, using the synthetic double-stranded oligonucleotides as competitors, to identify the site of binding of nuclear factors. Figure 4 shows the effects of a 100-fold molar excess of these competitors in the presence of nuclear extracts from wounded leaves. When the synthetic double-stranded oligonucleotide that corresponded to Box 1 was added, the retarded bands disappeared (lane 3). By contrast, retarded bands were still observed with the addition of competitors that corresponded to Box 2 and Box 3. This result indicates that nuclear factors bound specifically to the sequence that corresponded to Box 1.

Since Box 1 appeared to be a *cis*-element involved in the response to wounding from our binding assay *in vitro*, a chimeric gene –307/GUS with a disrupted CACGTG motif was constructed. This –289/GUS chimeric gene was introduced into tobacco plants. Figure 5 shows that no induction of GUS activity was observed in three independent transgenic plants with –289/GUS. Hence, expression of *prxC2* in response to wounding requires an intact Box 1 sequence.

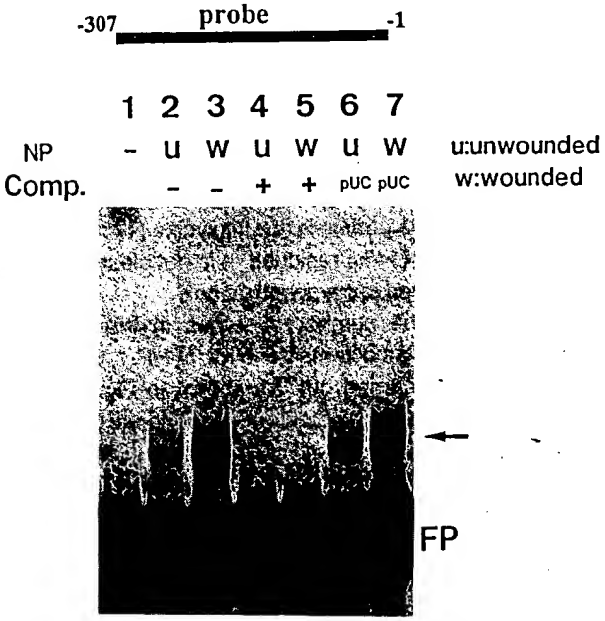

Figure 2. Interaction of nuclear factors with the promoter region of *prxC2*. The probe, ^{32}P-labeled DNA corresponding to the promoter region (–307 to –1) of the *prxC2* gene, was incubated in the absence (lane 1) and in the presence (lanes 2–7) of nuclear extracts (2 μg of protein) from unwounded leaves (lanes 2, 4 and 6) and wounded leaves (lanes 3, 5 and 7). As competitor DNA, homologous unlabeled DNA fragment (lanes 4 and 5) or pUC19 (lanes 6 and 7) was added to the reaction mixtures.

Isolation of cDNA that encodes the TFHP-1 protein

Tobacco nuclear proteins bound to Box 1 that contained the CACGTG motif. Total RNA was extracted from tobacco leaves which had been incubated in phosphate buffer for 24 h after wounding. We screened the tobacco

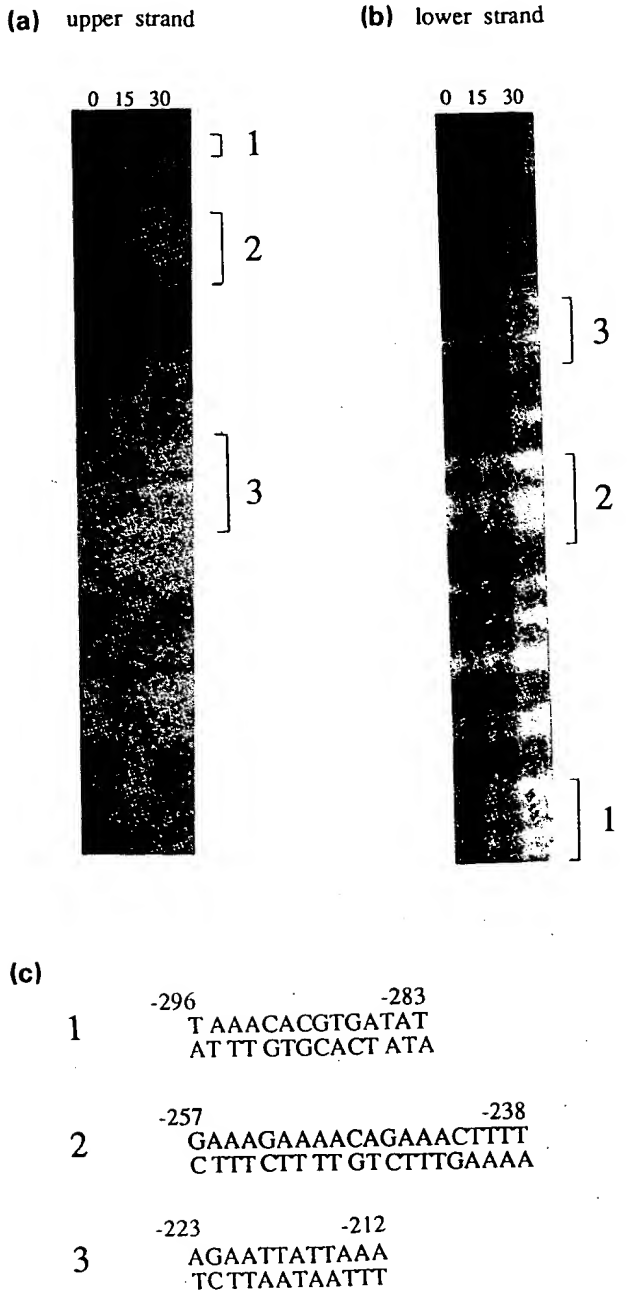

Figure 3. DNase I footprinting analyses.
The DNA fragment of the promoter region (–527 to –1) of *prxC2* was end-labeled with ^{32}P on the upper strand (a) or the lower strand (b). The DNA fragment was incubated without (0) or with a nuclear extract from wounded leaves (15 and 30 μg). The regions protected from enzymatic digestion are indicated (c).

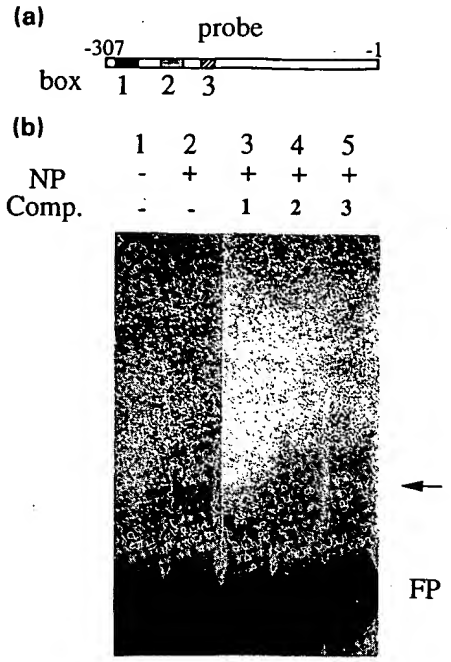

Figure 4. Competitive binding assay.
The binding activity was examined with nuclear extracts from wounded leaves. The labeled DNA fragment was incubated in the absence (lane 1) and in the presence (lanes 2–5) of a nuclear extract from wounded leaves. A 100-fold molar excess of synthetic double-stranded oligonucleotide was added (lane 3, Box 1; lane 4, Box 2; lane 5, Box 3).

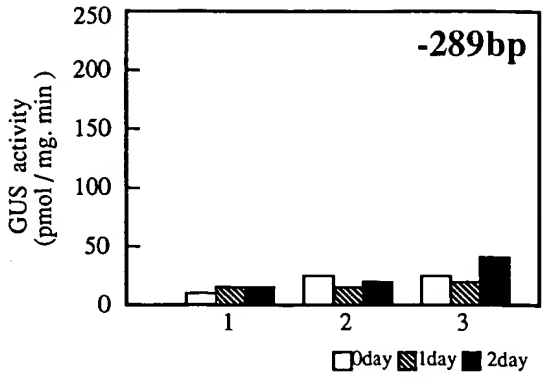

Figure 5. Wound-induced expression of *prxC2* (–289)/GUS in transgenic tobacco leaves.
Numbers on abscissa indicate individual transformants.

cDNA expression library derived from wounded tobacco leaves using a double-stranded oligonucleotide probe that corresponded to Box 1. One positive clone, 7e, was obtained from screening of 500 000 recombinant phages. The cDNA insert of 7e was subcloned into the SK(–) plasmid and the recombinant plasmid p7e was used for further experiments.

The nucleotide sequence of the cDNA insert was determined by the dideoxy chain-termination method. The insert DNA of 1 kbp encoded an open reading frame equivalent to 234 amino acids (Figure 6a). Since Northern blot analysis showed that the size of the mRNA was about 1 kb (see Figure 8a and b), the insert cDNA of 7e was probably full length. We designated the protein encoded by this cDNA TFHP-1.

Analysis of the deduced amino acid sequence showed that the encoded protein contains three leucine residues (nos 152, 159 and 166) arranged as heptad repeats. In addition, several basic amino acids were located adjacent to the upstream of the leucine repeats. These two structural motifs, the basic domain and the leucine repeats, are characteristic features of a class of transcription factors referred to as bZip proteins (Vinson *et al.*, 1989). However, the basic region of the TFHP-1 protein showed low homology to other plant bZip proteins, such as GBF-1, TAF-1 and HBP-1a (Oeda *et al.*, 1991; Schindler *et al.*, 1992; Tabata *et al.*, 1991).

Secondary-structural analysis of the predicted TFHP-1 protein indicated that amino acids 118–166 formed an α-helix followed by a turn or loop and a second α-helical region. These helices appear somewhat similar to the second helix of the helix–loop–helix (HLH) family of transcription factors (Figure 6a); (Benezra *et al.*, 1990). The TFHP-1 protein showed no homology to the first helix of the HLH family. Though the TFHP-1 protein contains several basic residues upstream of the helices, it lacks the highly conserved basic residues immediately upstream of the HLH region that are present in members of the HLH family and can activate transcription (Davis *et al.*, 1990; Voronova and Baltimore, 1990). However, the second helix exhibited homology to the HLH family of transcription factors (Figure 6b). The amino-terminal region (residues 1–30) of the TFHP-1 protein is rich in glycine (17%), serine (14%) and alanine (14%) residues. It is not clear whether this region is involved in *trans*-activation.

To determine the number of genes in the tobacco genome that are related to the gene for *TFHP-1*, Southern hybridization was carried out. As shown in Figure 8(a), one hybridizing band was obtained with genomic DNA that has been digested with either *Eco*RI or *Bam*HI. This result suggests that *TFHP-1* is a single-copy gene.

DNA-binding specificity of TFHP-1 protein

In order to determine whether the TFHP-1 protein could indeed bind to the Box 1 sequence, we prepared extracts from *Escherichia coli* carrying the expression vector pSK(–) before and after induction of IPTG. The extracts were tested with a synthetic double-stranded oligonucleotide fragment that corresponded to Box 1 as probe in gel-retardation assays. Figure 7(a) shows that a DNA–

(a)

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TCAAAAGCTTGACAAAAATGGCCAGCTATTCTCTTCCATCACCAATAGCCAAAAGGCTAG    60
                  M  A  S  Y  S  L  P  S  P  I  A  K  R  L  E    15

AAGGTAAAGTAGCACTAATAACTGGTGGAGCTAGCGGCATAGGAGCCGCCACAGCTAGGC   120
  G  K  V  A  L  I  T  G  G  A  S  G  I  G  A  A  T  A  R  L    35

TTTTTGTTCAACATGGTGCAAAGGTTACAATTGCAGACATTCAAGACAACCTTGGAACTT   180
  F  V  Q  H  G  A  K  V  T  I  A  D  I  Q  D  N  L  G  T  S    55

CTTTAGTACAAGAAATTGGCAACGAACATACAATCTTTATCCATTGCAATGTTGCTGTTG   240
  L  V  Q  E  I  G  N  E  H  T  I  F  I  H  C  N  V  A  V  E    75

AATCAGACGTTCAAAATGTTGTAGATGCAACAATTGCCAAATTTGGTAAGCTGGACATAA   300
  S  D  V  Q  N  V  V  D  A  T  I  A  K  F  G  K  L  D  I  M    95

TGTTTCAGTACGCTGGTATAGGAGGTAAGTCAATTTCCAGCATCTTAGATGTCGTACGAA   360
  F  Q  Y  A  G  I  G  G  K  S  I  S  S  I  L  D  V  V  R  I   115

TAATTAAGACAGTGTTCGATGTAAACATTGTTGGCGCCTTCTTTTGCGCTAAGCATGCTG   420
  I  K  T  V  F  D  V  N  I  V  G  A  F  F  C  A  K  H  A  A   135

CTAGAGTAATGATTCCATTCAAGAAAAGGTTCCATTATCTTCACAGCAAGTTATTGTCCC   480
  R  V  M  I  P  F  K  K  R  F  H  Y  L  H  S  K  L  L  S  R   155

GCACGCATATTCTGCATCAAAAAGGTGCAGTTTTGGGATTTTCCAAGAACATTGGGGTCG   540
  T  H  I  L  H  Q  K  G  A  V  L  G  F  S  K  N  I  G  V  E   175

AATTAGGGAAGTACGGAATAAAAGTTAACTGTGTTTCTCCTCATTACATTAGCACACCAC   600
  L  G  K  Y  G  I  K  V  N  C  V  S  P  H  Y  I  S  T  P  L   195

TTGTATTGAATGCGCTTGGAATAGCTGAGAGAGAGATAGCGGAAAAATGGTTTGCAGCAG   660
  V  L  N  A  L  G  I  A  E  R  E  I  A  E  K  W  F  A  A  G   215

GAGGAAATTTGAAAGGAGCTTTACTAGATGAAGAGGAGGTCGCAAAGGCAGTGCTATCTT   720
  G  N  L  K  G  A  L  L  D  E  E  E  V  A  K  A  V  L  S  *   234

AGCAAGTGATGATTCTAAATATGTGAGTGGTATGAATCTGGTTATTGATGGTGGTTTTAG   780

TACCACAAATGTGGCTTAACAGAGGCCTACAACAAATTGTTCTCATCCGAAAATACCCAT   840

CAGTGTTGATTTTTTCAAAATGAGTGGAATGAATAAAGAGGACTAACTAAATGATTATAT   900

CAGATCCCTCAACTGAAAACTTTCCAATGTGCAATATACAAGGTAAGGATAAGAGGCCAA   960

TGATATCTATAGTAGTTTTTAAAAAAAAAAAAAAAAAA                         998
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(b)

Helix-2 sequence:

LLSRTHILHQKGAVL	TFHP-1
PQTKLLILHQAVAVI	E12 (rat)
RLPKVEILRNAIRYI	myoD (mouse)
KVSKVEILQHVIDYI	Id (mouse)
KLSKVDTLRIAVEYI	achaete sc. (Dros.)

Figure 6. Nucleotide sequence and deduced amino acid sequence of *TFHP-1* and homology to the helix–loop–helix class of transcription factors.
(a) The sequence of the HLH motif predicted by the Robson–Garnier secondary-structure algorithm (Garnier *et al.*, 1978) is denoted by arrows, the basic region is shown in bold type and leucine repeats are underlined.
(b) Comparison of the second helix sequence between *TFHP-1* and members of the HLH family. Sources of data: *TFHP-1* (this study); E12 (Voronova and Baltimore, 1990); myoD (Davis *et al.*, 1990); Id (Benezra *et al.*, 1990) and achaete sc (Villares and Cabrera, 1987).

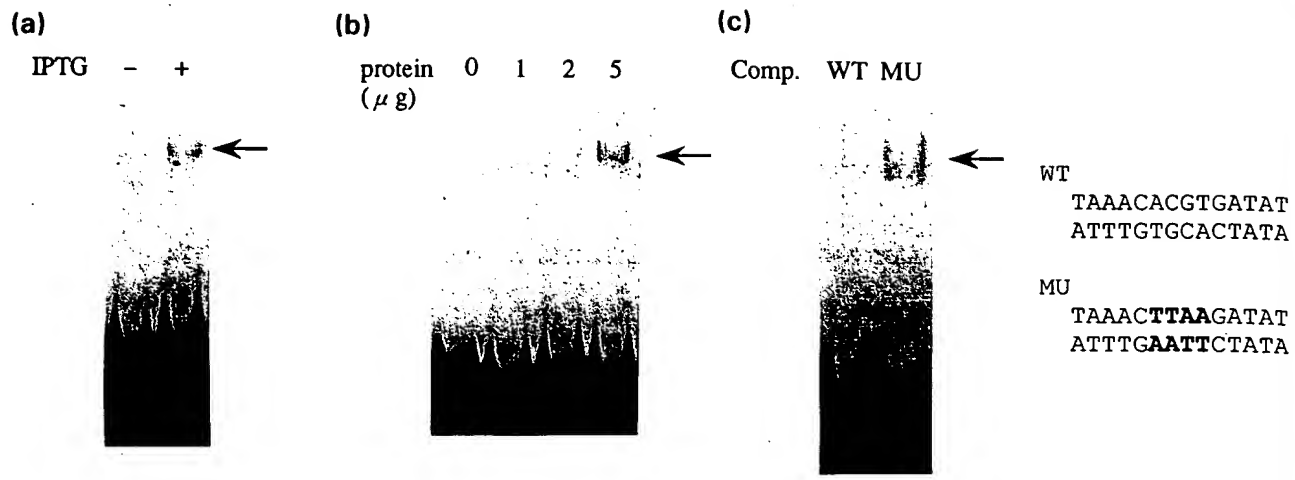

Figure 7. Interaction of TFHP-1 protein with the Box 1 sequence. ^{32}P-labeled DNA corresponding to the Box 1 sequence was used as probe.
(a) Cultures of exponential phase were induced with 2 mM IPTG (+); uninduced cultures were used as controls (–).
(b) Variations in the prevalence of the DNA–protein complex at different concentrations of protein.
(c) Effect of wild-type and mutant competitors. A 100-fold molar excess of competitor was added.

protein complex was present when extracts were prepared from IPTG-induced cells, and the amount of the DNA–protein complex increased in proportion to the concentration of the extracts (Figure 7b). To determine whether the DNA–protein complex resulted from a specific interaction with Box 1, we performed a competition assay using a synthetic mutant oligonucleotide. Figure 7(c) shows that the DNA–protein complex disappeared upon the addition of the wild-type competitor. However, the mutant competitor, in which the CACGTG motif had been disrupted, could not eliminate the retarded band. These results indicate that the TFHP-1 protein can bind specifically to the Box 1 sequence.

Pattern of expression of TFHP-1 mRNA

Total RNA was extracted from tobacco roots, stems and leaves. Twenty micrograms of total RNA were subjected to electrophoresis and blotted, and the level of expression of the TFHP-1 mRNA in each organ was determined by Northern hybridization (Figure 8b). This mRNA was abundant in roots and stems. mRNA of *TFHP-1* was also detected in leaves.

Next, we extracted total RNA from tobacco leaves that had been incubated in phosphate buffer for several hours after wounding to determine whether the transcript from the *TFHP-1* gene was induced by wounding. mRNA transcribed from the *TFHP-1* was observed at 0 h (before wounding) and its level remained almost constant for 24 h (Figure 8c). These results indicate that the *TFHP-1* gene is transcribed constitutively in tobacco.

Effect of TFHP-1 *on expression of* prxC2

To see whether the recombinant TFHP-1 could function as a transcription activator *in vivo*, we constructed plasmids containing antisense- and sense-*TFHP-1* under the control of CaMV 35S promoter as effector. Transient-expression assay in tobacco protoplasts after electroporation was carried out on co-introductions of the effector and reporter of *prxC2* (–529)/GUS. At 24 h after the introduction, GUS activities were measured. Co-introduction of sense-*TFHP-1* did not increase GUS activity compared with introduction of only reporter (data not shown). However, the GUS activities of reporter were decreased dependent on the amount of the effector of antisense-*TFHP-1* (Figure 9). When pBluescriptSK(–) was introduced as non-specific effector, into tobacco protoplasts, GUS activity of reporter was the same as when reporter alone was introduced. Therefore, TFHP-1 protein is associated with expression of *prxC2* and may act as a transcription factor.

Discussion

Peroxidases have been implicated in the responses of plants to physiological stress and pathogens. We have cloned four genomic DNAs that encode HRP. Among them, *prxC2* was strongly expressed upon wounding in horseradish leaves and the expression of *prxC2* was found to be regulated at the transcriptional level (Kawaoka *et al.*, 1994). To obtain more information about the functional organization of the *prxC2* promoter, we constructed four promoter–GUS fusions with promoters of different

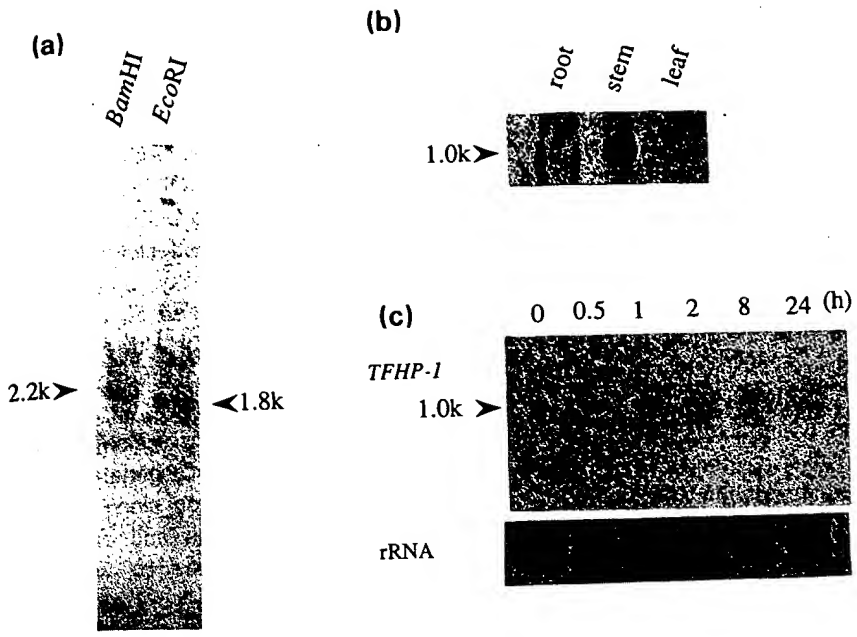

Figure 8. Genomic Southern and Northern blot analysis of *TFHP-1*.
(a) Genomic Southern blot analysis of *TFHP-1*. Genomic DNA extracted from tobacco leaves was digested with *Bam*HI and *Eco*RI.
(b) Organ-specific expression of *TFHP-1*. Total RNA was extracted from roots, stems and leaves.
(c) Wound-induced expression of *TFHP-1*. Tobacco leaves were cut into small pieces and incubated in phosphate buffer. Total RNA was extracted at indicated times after wounding. Wheat ribosomal DNA was used as internal control.

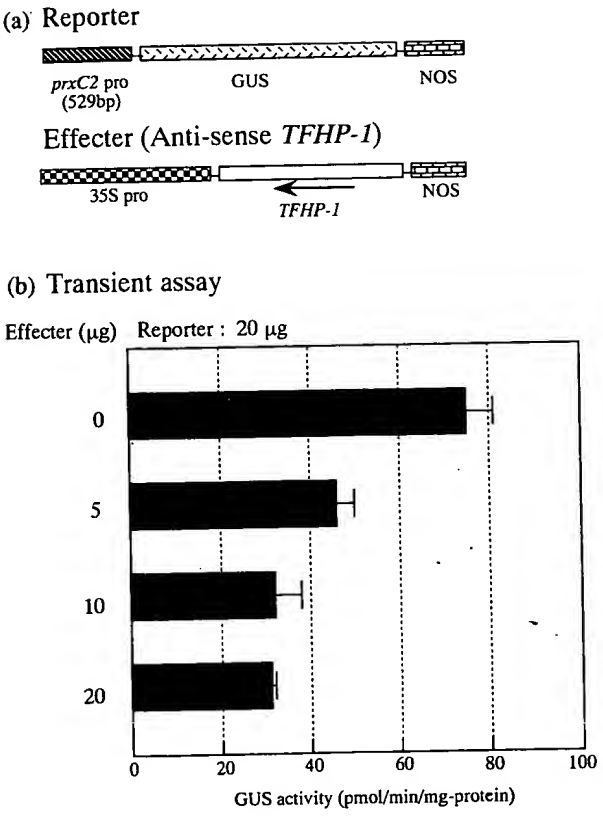

Figure 9. Effect of antisense-*TFHP-1* on a transient of *prxC2*.
(a) Plasmid constructs. Plasmids used are described in Experimental procedures.
(b) Transient expression of *prxC2* (–529)/GUS with antisense-*TFHP-1*. Reporter (20 μg) with effector (0–20 μg) were introduced into tobacco protoplasts by electroporation. GUS actvity in extracts of protoplasts was measured after incubation for 24 h.

lengths and determined whether the specific regulatory properties of the promoter were changed by the deletions. Some variations in GUS activity were found among individual plants that had been transformed with each construct, probably as a result of positional effects of insertion of the transgene at different sites in the genome or differences in numbers of copies of the introduced gene. Deletion of the promoter from –1035 to –307 allowed induction of GUS activity by wounding in three independent transformants (Figure 1). However, no induction was observed with the –99 construct, in which the CAAT box had been deleted. Therefore, the *cis*-elements required for induction of *prxC2* by wounding appeared to be located between positions –307 and –99.

Gel retardation assays revealed the presence of nuclear factors that bound specifically to the DNA fragment from –307 to –1 of the promoter region of *prxC2* (Figure 2). The retarded bands of nuclear factor–DNA complexes, formed in the presence of nuclear extracts from wounded leaves, were more intense than those from unwounded leaves. This result indicates that the binding activity of the nuclear factors was stimulated by wounding, perhaps by regulation of the activation of the nuclear factors at the post-transcriptional level, by modifications such as phosphorylation (Klimczak *et al.*, 1992). Since fragments longer than 300 bp were used as probes, the retarded bands were not well separated and some of the bands were unclear. DNase I footprinting revealed the presence of three regions that were protected from enzymatic digestion (Figure 4). Competitive binding assays

showed that the retarded bands disappeared during reaction with a synthethic double-stranded oligonucleotide that corresponded to Box 1. Box 1 contains the sequence CACGTG, which has been shown to control induction by UV light of chalcone synthase (Staiger *et al.*, 1989) and is a coremotif of the so-called G-box (Schindler *et al.*, 1992), and it is also very similar to the abscisic acid (ABA)-responsive element (Guiltinan *et al.*, 1990). Thus, the sequence CACGTG is highly conserved in several genes and is a common *cis*-element involved in responses to environmental stress, such as wounding or UV light. Sequences containing the G-box like motifs might have structures that allow nuclear factors to bind easily *in vitro*. In our binding assay *in vitro*, nuclear factors bound to the Box 1 sequence. Therefore, we constructed a chimeric gene, –289/GUS, with a disrupted Box 1. No induction by wounding of GUS activity was found (Figure 5). These results are supported by the results of the binding assay *in vitro* and, thus, Box 1 sequence appeared necessary for expression of *prxC2* in response to wounding.

The multiplicity of G-box-binding factors has been confirmed by molecular cloning studies. To date, several cDNA clones have been isolated that encode proteins that interact specifically with sequences that include CACGTG. These cDNA clones include *Arabidopsis* GBF-1 (Schindler *et al.*, 1992), wheat EmBP-1 (Guiltnan *et al.*, 1990), tobacco TAF-1 (Oeda *et al.*, 1991) and parsley CPRF-2 1,2,3 (Weisshaar *et al.*, 1991). Then, we screened a cDNA expression library from wounded tobacco leaves and isolated one positive clone. Nucleotide sequence analysis of this cDNA clone, p7e, revealed that it encoded a protein of 234 amino acid residues, designated TFHP-1 and that this putative protein contains a basic domain and leucine repeats at its carboxy terminus. This bipartite structure is characteristic of the bZip proteins (Vinson *et al.*, 1989). However, the TFHP-1 protein displayed low homology to the basic domain of members of the bZip family. Secondary structural analysis of the TFHP-1 protein revealed a HLH structure. The second helix exhibits high homology to the HLH family of transcription factors (Figure 6b). However, no similarity was apparent to the highly conserved basic residues immediately upstream of the HLH region in other proteins. It has been reported that the product of the c-*myc* gene (Myc) has bHLH and Zip domains, and binds to the CACGTG motif (Blackwell *et al.*, 1990; Blackwood and Eisenman 1991). TFHP-1 protein has low homology to the Myc. These observations suggest that TFHP-1 is the first member of a novel class of proteins that specifically bind to the CACGTG motif.

Tobacco bZip proteins, TGA1a and TAF-1, have been reported to function as *trans*-activators in plants (Oeda *et al.*, 1991; Yamazaki *et al.*, 1990). We examined whether the TFHP-1 protein could function as a *trans*-activator in a transient expression assay using tobacco protoplasts. DNA fragments containing sense- and antisense-*TFHP-1* under the control of CaMV 35S promoter as effectors and *prxC2* (–529)/GUS as reporters were co-introduced into tobacco protoplasts by electroporation. Although GUS activity in control protoplasts which had only reporter introduced was at a high level, no enhanced GUS activity was observed on co-introduction with the effector of sense-*TFHP-1* (data not shown). This result may be caused by the fact that protoplasts were in a 'wounded' condition and *prxC2* promoter induced without effector. However, antisense-*TFHP-1* suppressed expression of reporter (Figure 9). Therefore, TFHP-1 protein is associated with expression of the *prxC2* and may act as a transcription factor. If the TFHP-1 protein is to activate the *prxC2* gene, it should undergo post-transcriptional modification since TFHP-1 mRNA was expressed in each organ and enhanced expression of mRNA was not induced by wounding (Figure 8).

This study suggests that the Box 1 sequence, which contains the CACGTG motif as a G-box, is essential for the wound-induced expression of *prxC2*. A CACGTG motif has been reported to be present in the promoter regions of many plant genes (Williams *et al.*, 1992) and several proteins that bind specifically to the CACGTG motif have been isolated, as described above. However, the expression of such genes is independently regulated. Fundamental questions remain to be solved, in particular with respect to the specific signal-transduction pathways to each G-box-binding protein.

Experimental procedures

Plant material

Nicotiana tabacum cv. Petit Havana SR-1 was used in all gene transfer experiments. Tobacco plants were grown in a greenhouse at 25°C under continuous light.

Construction of chimeric genes

Construction of chimeric genes was performed basically by the method of Maniatis *et al.* (1982). Chimeric genes for each deletion from –1035 to –99 bp of the 5'-non-coding region and structural gene for GUS were constructed as described previously (Kawaoka *et al.*, 1992). A –289/GUS was constructed as described below. The –307/GUS construct inserted in pBI101 (Jefferson *et al.*, 1987) was digested with *Hin*dIII which acts as the cloning site and *Pma*CI which digests the sequence CAC ↓GTG at the site indicated by the arrow. After treatment with Klenow fragment, the blunt-ended fragment was self-ligated. The resulting sequence was AAGCTGTGATA^{-284}. The binary vectors harboring various chimeric genes were transferred from *Escherichia coli* strain HB101 into *Agrobacterium tumefaciens* strain LBA4404 by a triparental mating procedure with the helper plasmid pRK2013 (Ditta *et al.*, 1980).

Transformation of tobacco

Tobacco was transformed with *A. tumefaciens* LBA4404 that carried a modified form of pBI101 by using the leaf disk-infection (Rogers *et al.*, 1986). Transformants were selected in the presence of 100 μg ml^{-1} kanamycin and regenerated plants were grown in a greenhouse at 25°C.

Assay for GUS activity

GUS activity was assayed in T1 plants 3–4 weeks after regeneration of roots by the method described by Jefferson *et al.* (1987), with 4-methyl-umbelliferyl glucuronide as the substrate. GUS activity was expressed as picomoles of methyl-umbelliferone per minute per mg of protein. Protein was quantitated by the method of Bradford (1976).

Preparation of nuclear extracts

Nuclei were prepared from well-expanded tobacco leaves as described by Staiger *et al.* (1989). Tobacco leaves were cut into small pieces (about 3 mm). The pieces were immediately frozen in liquid nitrogen (unwounded) or were incubated in 10 mM sodium phosphate buffer (pH 7.0) at 25°C for 24 h under continuous light (wounded).

Preparation of probes and competitor DNAs

*Hin*fI–*Xba*I fragment from –307 to –1 of the 5'-non-coding region of *prxC2* was labeled with T4 DNA polymerase and [α-^{32}P]dCTP and purified by polyacrylamide gel electrophoresis, elution and ethanol precipitation. This fragment was used as the probe for gel-retardation assay.

The *Xba*I fragment from –529 to –1 of the 5'-non-coding region in *prxC2* was subcloned into pUC19 as the probe for DNase I footprinting. This plasmid was linearized by cleavage at one end of the insert by either *Hin*dIII or *Bam*HI. The staggered ends were filled in by the Klenow fragment of DNA polymerase in the presence of [α-^{32}P]dCTP. Following extraction with a mixture of phenol, chloroform and isoamylalcohol (25:24:1, v/v), and ethanol precipitation, the insert DNA was released from the vector DNA by digestion with either *Kpn*I or *Pst*I. Insert DNA was purified by polyacrylamide gel electrophoresis, elution and ethanol precipitation.

Oligonucleotides that corresponded to the presumptive binding sites, namely, Box 1 (–296 to –283), Box 2 (–257 to –238) and Box 3 (–223 to –212), were chemically synthesized on a Cyclone Plus DNA Synthesizer (MilliGen/Bioresearch). The oligonucleotides were annealed together and used as competitor DNAs. The synthetic double-stranded oligonucleotide probe was labeled with T4-polynucleotide kinase.

Gel-retardation assay and DNase I footprinting

The binding reaction was carried out in a 10 μl reaction mixture that contained 0.5 ng of labeled DNA probe, 2 mg of protein as nuclear extract, 12.5 mM Tris–HCl (pH 7.8), 5 mM EDTA, 100 mM KCl, 10% glycerol, 1 μM PMSF, 0.5 mM DTT and 2–5 μg of poly(dI–dC) (Pharmacia LKB Biotechnology). Reactions were allowed to proceed for 30 min on ice. Each reaction mixture was loaded without addition of dye on to a 4% non-denaturing polyacrylamide gel in 1× TAE (40 mM Tris–acetate, pH 8.0, 2 mM EDTA). Gels were run at approximately 10 V cm^{-1}. Following electrophoresis, each gel was dried and exposed to X-ray film at –70°C with an intensifying screen for 12–24 h. For the competitive binding assays, 50–100 ng of unlabeled DNA fragment were added to the reaction mixture.

The reactions for the DNase I footprinting assay were exactly the same as those for gel-retardation assays, with the exception that the footprinting reactions contained 15–30 ng of nuclear extract from wounded leaves. After the binding reaction, 2 μl of a solution of DNase I (60 u ml^{-1}) were added and the mixture was incubated for 60 sec at 20°C. The reaction was stopped by the addition of 90 μl of 6.25 m EDTA, 0.125% SDS, 375 mM sodium acetate and 62.5 μg ml^{-1} tRNA (Green *et al.*, 1988). The reaction mixture was extracted with an equal volume of a mixture of phenol, chloroform and isoamyl alcohol (25:24:1, v/v) and DNA was ethanol-precipitated. Pellets were resuspended in 95% deionized formamide that contained 10 mM EDTA (pH 8.0), 0.025% xylene cyanol and 0.025% bromophenyl blue, boiled for 3 min and loaded on to 4% denaturing polyacrylamide gels in 1× TBE (89 mM Tris–borate, 2 mM EDTA). After electrophoresis, the gel was dried and exposed to X-ray film.

Screening of the library and sequencing

Total RNA was isolated from tobacco leaf tissue that had been incubated in phosphate buffer (pH 7.0) for 24 h after wounding. Using oligo(dT) as primer, a cDNA library was constructed in λZAPII vectors (Stratagene). The amplified library was screened with a^{32}P-labeled double-stranded oligonucleotide fragment that corresponded to Box I between –296 and –283 bp. We used essentially the same screening protocol as Singh *et al.* (1988). Sequence analysis by dideoxy chain-termination (Sanger *et al.*, 1982) was performed on double-stranded DNA templates, using Sequenase™ (United States Biochemical). Sequence data were analyzed with the DNASIS programs (Hitachi).

Southern and Northern analyses

Genomic DNA was isolated from tobacco leaves, and Southern blot analysis was performed as described by Maniatis *et al.* (1982). Total RNA was extracted from tobacco tissue (Chomczynski and Sacchi, 1987), fractionated on a formaldehyde–agarose gel and blotted on to a nylon membrane (Hybond N; Amersham). The membrane was hybridized with ^{32}P-labeled probe in 50% formamide, 6× SSC, 1× Denhardt's solution, 0.5% SDS and 500 μg ml^{-1} salmon testis DNA at 42°C, then it was washed with 0.2× SSC, 0.1% (w/v) SDS at 42°C. Finally, the membrane was exposed to X-ray film. Wheat ribosomal DNA (Apples and Dovorak, 1982) were used as internal control.

Expression of TFHP-1 *in* Escherichia coli

E. coli cells carrying the expression vector SK(–) were grown to an absorbance at 660 nm of 0.5 in LB broth; isopropyl-β-D-thiogalactopyranoside was added to the culture at a final concentration of 1 mM and the cells were cultured for an additional 2 h. Cells were harvested by centrifugation and resuspended in 1/20 volume of buffer A (25 mM HEPES (pH 9.0), 1 mM EDTA, 1 mM PMSF, 1 mM DTT and 0.15% Nonidet P-40). After freezing of cells in liquid nitrogen and thawing on ice, lysozyme was added to a final concentration of 0.5 mg ml^{-1}, and the suspension of cells

was incubated on ice for 15 min. Then, KCl was added to a final concentration of 1.3 M. The lysates were gently rotated at 4°C for 15 min and they were centrifuged at 350 000 ***g*** for 45 min. The supernatant was dialyzed against buffer A with 100 mM KCl and 20% glycerol. Dialyzed samples were stored at –70°C.

Plasmid construction and electroporation

*Not*I fragment of *TFHP-1* was recloned into pSK(–) at the same site. The clones which had antisense and sense orientations were checked by the restriction mapping. The *Bam*HI–*Sac*I fragments of antisense- and sense-*TFHP-1* were inserted into the same sites of pBI221 with CaMV 35S promoter. These constructs were used as effectors. A chimeric gene of *prxC2* (–529)/GUS which was used as a reporter, was constructed as described previously (Kawaoka *et al.*, 1992). Electroporation of tobacco protoplast was carried out as described previously (Kawaoka *et al.*, 1992). GUS activity in extracts was measured after incubation for 24 h.

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The nucleotide sequence data reported in this paper will appear in the GSDB, DDBJ, EMBL and NCBI nucleotide sequence databases with the following accession number: D29976 (*TFHP-1*).